Quantitative test kit for NAD-dependent histone deacetylase activity

CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit

100 Assays

Cat# CY-1151

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Intended Use

The CycLex Research Product **CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit** detects SIRT1/Sir2 activity in lysates. Primarily, the CycLex Research Product **CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit** is designed for the rapid and sensitive evaluation of SIRT1/Sir2 inhibitors or activators using crude SIRT1/Sir2 fraction or purified SIRT1/Sir2. Additionally, any cultured primary cell, cell line, or tissue homogenate can be assayed for SIRT1/Sir2 activity with the CycLex Research Product **CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit** if the appropriate antibody direct against SIRT1 or Sir2 is used for immunoprecipitation.

Applications for this kit include:

1) Monitoring the purification of SIRT1/Sir2.
2) Screening inhibitors or activators of SIRT1/Sir2.
3) Detecting the effects of pharmacological agents on SIRT1/Sir2.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store recombinant SIRT1 at -70°C and all other components below -20°C.
- Don't expose reagents to excessive light.

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Introduction

Sir2 is a conserved protein and was recently shown to regulate lifespan extension both in budding yeast and nematode. In 2000, it was reported that the yeast Sir2 protein is a NAD(+)-dependent histone deacetylase that plays a critical role in transcriptional silencing, genome stability and longevity. A human homologue of Sir2, SIRT1, also functions as a NAD(+)-dependent-p53 deacetylase as well as a NAD(+)-dependent histone deacetylase. SIRT1 was shown to regulate the activity of the p53 tumor suppressor and inhibits apoptosis. These results have significant implications regarding an important role for SIRT1 in modulating the sensitivity of cells in p53-dependent apoptotic response and the possible effect in cancer therapy. Since the function of p53 is made to strengthen powerfully by using together with DNA damaging reagent, it is expected that inhibitor of SIRT1 becomes an effective anticancer drug.

However, the conventional method for measuring SIRT1/Sir2 activity is very complicated and laborious. In order to measure SIRT1/Sir2 enzyme activity, it is necessary to prepare radioactive acetylated histone as a substrate. First, cells have to be labeled metabolically with radioactivity by adding radioactive acetic acid to the culture medium. Second, radioactive acetylated histone has to be purified from the cells. Following the reaction, it is necessary to extract and separate the radioactive acetyl group, which has been released from acetylated histone, using ethyl acetate to measure the activity of the enzyme based on the radioactivity.

Although a method for measuring the activity of deacetylase without the use of radioactive substances was reported in recent years, owing to the use of fluorescent-labeled acetylated lysine as a substrate, the reaction product must be separated from the intact substrate and the fluorescent intensity measured by reverse phase HPLC. As mentioned above, these measurement systems are difficult to adapt for processing many samples under a variety of conditions, because of their complicated operation. Thus a simple system for biochemical analysis as well as for inhibitor screening without the use of radioactive substances is preferred.

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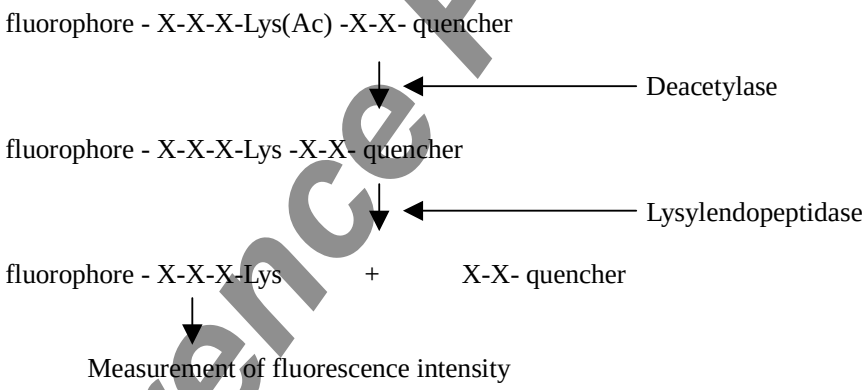
Principle of the Assay

CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit measures the activity of SIRT1/Sir2 by the basic principle of changing a SIRT1/Sir2 reaction into the activity of the protease. In order to measure the enzyme activity of SIRT1/Sir2, which is the NAD dependent Histone deacetylase, and its homolog, this kit is designed so that the activity of NAD dependent Histone deacetylase can be measured under existence of Trichostatin A, which is the powerful inhibitor of HDACs.

In this kit, fluorophore and quencher are coupled to amino terminal and carboxyl terminal of substrate peptide, respectively, and before reaction of deacetylase, the fluorescence cannot be emitted. However, if SIRT1/Sir2 performs deacetylation, substrate peptide will become cut by the action of protease added simultaneously, quencher will separate from fluorophore, and fluorescence will be emitted. Deacetylase enzyme activity is measured by measuring this fluorescence intensity.

Since it is very simple to measure and it can be performed at a low price, the measurement of SIRT1/Sir2 activity in most laboratories is possible if they are equipped with a fluorescent reader for microtiter plates. Considering that the use of fully automatic apparatus to measure fluorescence intensity has become widespread, SIRT1/Sir2 activity measurement, which could not be made by the conventional method, is now possible with the CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit using the same equipment. This new method of measurement should dramatically raise the efficiency of inhibitor screening and biochemical analysis of these enzymes.

Measuring Principle of The CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit



***Note: This measuring principle and kit are covered under CycLex's patents.**
U.S. Patent No. 7,033,778 and No. 7256013
European Patent No. 1243658
Japanese Patent No. 4267043
Canadian Patent No. 2392711

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Materials Provided

Each kit contains

Materials	Quantity	Storage
① 10X SIRT1 assay buffer	1 ml x 2	Below -20°C
② 50X Fluoro-Substrate Peptide (1 mM)	100 µ l x 1	Below -20°C
③ 50X Fluoro-Deacetylated Peptide (1 mM)	50 µ l x 1	Below -20°C
④ Lysylendopeptidase (100 mAU/ml)	50 µ l x 1	Below -20°C
⑤ 100X NAD (20 mM)	100 µ l x 1	Below -20°C
⑥ 200X Trichostatin A (0.2 mM)	100 µ l x 1	Below -20°C
⑦ Recombinant SIRT1	200 µ l x 1	-70°C
⑧ 100X Stop solution	100 µ l x 1	Below -20°C
⑨ Instruction manual	1	room temp.

Materials Required but not Provided

- **Microplate for fluorometer**
- **Microplate reading fluorometer** capable of excitation at a wavelength in the range 340-360 nm and detection of emitted light in the range 440-460 nm.
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1000 µL precision pipetors with disposable tips.
- **multi-channel pipette**
- **Microplate shaker**
- **Deionized water of the highest quality**
- **500 or 1000 mL graduated cylinder**
- **Reagent reservoirs**

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Precautions

- Please thaw ②50X Fluoro-Substrate Peptide and ③50X Fluoro-Deacetylated Peptide at room temperature before use. Then, thaw the other reagents in ice and use after they are completely thawed.
- Please avoid repeated freezing and thawing of the ⑦Recombinant SIRT1 in this kit. There is a possibility that the enzyme activity may be inactivated. Aliquot to 10-20 µL and store at –70°C
- Please avoid mixing of protease inhibitors such as PMSF, or alkyl amine in the sample that will be measured SIRT1/Sir2 activity.
- Do not use kit components beyond the indicated kit expiration date.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- Do not mouth pipet or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**

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Detailed Protocol

Description of assay system

CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit can measure the enzyme activity of SIRT1/Sir2 with a homogeneous method. In this method, the reaction is initiated and the fluorescence intensity is measured by mixing simultaneously fluorescence-labeled acetylated peptide, which is substrate, SIRT1/Sir2, trichostatin A, NAD and lysylendopeptidase. Since the reaction is not stopped, it is necessary to measure fluorescence intensity at regular intervals after the reaction is initiated, and to determine reaction velocity. Alternatively, within a time in which the reaction velocity is kept constant, it is also possible to stop the reaction by adding 2X stop solution and to measure fluorescence intensity.

Preparation Method for Assay Reagents

Thaw ②50X Fluoro-Substrate Peptide and ③50X Fluoro-Deacetylated Peptide at room temperature. Stand other reagents in ice to thaw. Use them after they thaw completely.

#1. 1X SIRT1 assay buffer (50 mM Tris-HCl (pH 8.8), 0.5 mM DTT)
Quantity Required: 50 µL/assay
・Dilute the ①10X SIRT1 assay buffer 1:10 with distilled water.
Since this is the base buffer for the assay, prepare 1 vial (1 ml) of ①10X SIRT1 assay buffer mixed with 9 ml of dH_2O and store SIRT1 assay buffer at 4°C.

#2. X20 diluted Lysylendopeptidase (5 mAU/ml)
Quantity required: 2.5 µL/assay
・Dilute the ④Lysylendopeptidase 1:20 with #1. 1X SIRT1 assay buffer.

#3. 10X NAD (2 mM β-NAD)
Quantity required: 5 µL/assay
・Dilute the ⑤100X NAD 1:10 with #1. 1X SIRT1 assay buffer.

#4. 10X TSA (10 µM)
Quantity required: 5 µL/assay
・Dilute the ⑥200X Trichostatin A 1:20 with #1. 1X SIRT1 assay buffer.

#5. 10X Test sample (10X final concentration, e.g. a candidate of inhibitor or activator)
Quantity Required: 5 µL/assay
・Dilute Test sample to 10X final desired concentration with #1. 1X SIRT1 assay buffer.

#6. X5 diluted recombinant SIRT1
Quantity Required: 10 µL/assay
・Dilute the ⑦Recombinant SIRT1 1:5 with #1. 1X SIRT1 assay buffer.
(Note! Use "#6. X5 diluted recombinant SIRT1" within the same day they are prepared.)

#7. 2X Stop solution
Quantity required: 50 µL/assay
・Dilute the ⑧100X Stop solution 1:50 with dH_2O.

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#8. SIRT1 reaction buffer (Final 50 mM Tris-HCl (pH 8.8), 0.5 mM DTT, 0.25 mAU/ml Lysylendopeptidase, 1 µM Trichostatin A and 20 µM Fluoro-Substrate Peptide in 50 µL of reaction mixture)

Quantity Required: 30 µL/assay (in case of adding 10 µL of "#6. X5 diluted recombinant SIRT1" and 5 µL of "#5. 10X Test sample")

・Mix following reagents (30 µL/1 assay)

1.	①10X SIRT1 assay buffer	5 µL
2.	②50X Fluoro-Substrate Peptide	1 µL
3.	#2. X20 diluted Lysylendopeptidase	2.5 µL
4.	#4. 10X TSA	5 µL
5.	dH_2O	16.5 µL
	Total	30 µL

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SIRT1/Sir2 Assay Procedures

1. Assay method

Assay reagents	Test sample	No enzyme control	No Test sample control	No NAD control
#8. SIRT1 reaction buffer **#3. 10X NAD** **#5. 10X Test sample** **dH_2O**	**30 µL** **5 µL** **5 µL** **-**	**30 µL** **5 µL** **-** **15 µL**	**30 µL** **5 µL** **-** **5 µL**	**30 µL** **-** **-** **10 µL**
#6. X5 diluted recombinant SIRT1 (or Your enzyme sample)	**10 µL**	**-**	**10 µL**	**10 µL**

1) Following the above table, add Reagent #3, #5 and #8 or dH_2O to each well of the microplate. Finally, initiate reaction by adding 10 µL of "#6. X5 diluted recombinant SIRT1" or "your enzyme sample" to each well and mixing thoroughly. Incubate at room temperature (Ca.25°C).

2) Read fluorescence intensity for 30 to 60 minutes at 1 to 2 minute intervals using microtiter plate fluorometer with excitation at 340-360 nm and emission at 440-460 nm. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Alternate procedure

1') Following the above table, add Reagent #3, #5 and #8 or dH_2O to each well of the microplate. Finally, initiate reaction by adding 10 µL of "#6. X5 diluted recombinant SIRT1" or "your enzyme" to each well and mixing thoroughly. Incubate at room temperature (Ca.25°C).

2') While the reaction rate is kept constant, add 50 µL of "#7. 2X Stop solution " to each well at appropriate time to stop the reaction, and measure fluorescence intensity in a microplate fluorescence reader with excitation at 340 nm and emission at 440 nm

3') The difference in fluorescence intensity between "**No Test sample control**" and "No enzyme control" indicates the SIRT1/Sir2 activity.

Note-1: It is possible to change the volume of assay reagents and sample as far as it sets up the final concentration of each reagents in a reaction mixture as indicated as below.

Note-2: Duplicate measurement is strongly recommended.

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2. Assay control

1. When the chemicals that have an inhibitory effect on lysylendopeptidase come to be mixed in SIRT1/Sir2 fraction purified from various cells or the immunoprecipitate using the specific antibody against SIRT1/Sir2 or other proteins, precise SIRT1/Sir2 enzyme activity cannot be measured. Since the protease inhibitors used in the usual protein purification process strongly inhibit lysylendopeptidase activity, please avoid using any protease inhibitors during the process of protein purification.

   If there is such a possibility, please carry out the experiment of **"Positive control"** and **"Assay control-1"** in the following table, using Fluoro-Deacetylated Peptide to reference. When Fluoro-Deacetylated Peptide is used, fluorescence intensity should increase whenever there is no SIRT1/Sir2 activity in your enzyme sample. When there is an inhibitory effect on lysylendopeptidase activity, even if there is SIRT1/Sir2 activity in a sample, fluorescence intensity should not increase.

2. Not only when an inhibitory effect on SIRT1/Sir2 is in test chemicals, but also when there is an inhibitory effect on lysylendopeptidase, final fluorescence intensity will not increase. Please use Fluoro-Deacetylated Peptide instead of Fluoro-Substrate Peptide, and please carry out the experiment of **"Positive control"** and **"Assay control-2"** that does not add SIRT1/Sir2 in the following Table. Although fluorescence intensity increases when Fluoro-Deacetylated Peptide is used, when an inhibitory effect on lysylendopeptidase activity occurs in a test chemicals, fluorescence intensity does not increase.

Assay reagents	Assay control-1	Assay control-2	Positive control
① 10X SIRT1 assay buffer	**5 µL**	**5 µL**	**5 µL**
③ 50X Fluoro-Deacetylated Peptide	**1 µL**	**1 µL**	**1 µL**
#3. 10X NAD	**5 µL**	**5 µL**	**5 µL**
#4. 10X TSA	**5 µL**	**5 µL**	**5 µL**
#5. 10X Test sample	**-**	**5 µL**	**-**
#6. X5 diluted recombinant SIRT1 (or Your enzyme sample)	**5 µL**	**-**	**-**
dH_2O	**26.5 µL**	**26.5 µL**	**31.5 µL**
#2. X20 diluted Lysylendopeptidase	**2.5 µL**	**2.5 µL**	**2.5 µL**

1) Following the table above, add Reagent ①, ③, #3, #4, #5 or #6 and dH_2O to each well. Finally, add 2.5 µL of "#2. X20 diluted Lysylendopeptidase" to each well and mix thoroughly to initiate reaction.

2) Incubate for 10 min or desired length of time at room temperature (Ca.25°C).

3) Add 50 µL of "#7. 2X Stop solution" to each well.

4) Read fluorescence intensity using microtiter plate fluorometer with excitation at 340 nm and emission at 440 nm.

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Cautions

1. In order to measure the activity of SIRT1/Sir2 correctly, it is necessary to conduct the control experiments for "**No enzyme control**" and "**No NAD control**" at least once in addition to "**No Test sample control**," as indicated in the above table of Assay method. Although fluorescence intensity increases in **"No Test sample control**" when SIRT1/Sir2 enzyme activity is in the sample, the increase in fluorescence intensity is not observed in "**No enzyme control**" and "**No NAD control**".

2. In order to estimate the inhibitory effect on SIRT1/Sir2 activity in the Test sample correctly, it is necessary to conduct the control experiment of "**No Test sample control**" at least once for every experiment and "**No NAD control**" at least once for the first experiment, in addition to "**Test sample**" as indicated in the above table of Assay method. When test chemicals cause an inhibitory effect on SIRT1/Sir2 activity, the level of increase of fluorescence intensity is weakened as compared with "**No Test sample control**". The increase in fluorescence intensity is not observed in "**No NAD control**".

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Troubleshooting

1. When chemicals that have an inhibitory effect on lysylendopeptidase are mixed in a crude SIRT1/Sir2 fraction purified from various cells or the immunoprecipitate using a specific antibody against SIRT1/Sir2 or other proteins, precise SIRT1/Sir2 enzyme activity cannot be measured. Since the protease inhibitors used in the usual protein purification process inhibit lysylendopeptidase activity strongly, please avoid the use of any protease inhibitors during the protein purification process.

2. Final fluorescence intensity will not increase, both when test chemicals have an inhibitory effect on SIRT1/Sir2, and also when there is an inhibitory effect on lysylendopeptidase.

3. If the test reagents themselves emit fluorescence at excitation wavelength: 340-360 nm and fluorescence wavelength: 440-460 nm, the inhibitory effect of the test assay cannot be evaluated correctly.

4. The recombinant SIRT1 should be run in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.

5. The reaction curve is nearly a straight line if the kinetics of the assay is of the first order. Variations in the protocol can lead to non-linearity of the curve, as can assay kinetics that are other than first order. For a non-linear curve, point to point or quadratic curve fit methods should be used.

6. Poor duplicates indicate inaccurate dispensing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for multi-channel pipettor maintenance.

Reagent Stability

All of the reagents included in the CycLex Research Product **CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, **store the ⑦Recombinant SIRT1 at -70°C**, all other kit reagents should be stored below -20°C.

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Sample Preparation

Numerous extraction and purification methods can be used to isolate SIRT1. The following protocols have been shown to work with a number of different cells and enzyme sources and are provided as examples of suitable methods. Crude samples can frequently be used without dilution while more concentrated or highly purified SIRT1 should be diluted. It is strongly advised that the user always perform an initial experiment to determine the proper dilution to be used in subsequent experiments. This need not be any more than a single time point assay using serial dilutions of the crude extract, cell lysate or sample fraction taken prior to a purification step. All sample preparation should be performed at 4°C and recovered fractions should be kept at -70°C to prevent loss of enzymatic activity.

Buffers

*Lysis Buffer:	*Sucrose cushion:	*Extraction buffer:
10 mM Tris-HCl (pH7.5) 10 mM NaCl 15 mM $MgCl_2$ 250 mM Sucrose 0.5 % NP-40 0.1 mM EGTA	30 % Sucrose 10 mM Tris-HCl (pH7.5) 10 mM NaCl 3 mM $MgCl_2$	50 mM Hepes-KOH (pH 7.5), 420 mM NaCl, 0.5 mM EDTA Na_2, 0.1 mM EGTA, 10 % glycerol.

Procedure

Isolation of Nuclei

1. Suspend 1x 10^7 cells (100 mm dish sub-confluent) into 1ml of Lysis buffer.
2. Vortex for 10 second.
3. Keep on ice for 15 min.
4. Spin the cells through 4 ml of sucrose cushion at 1,300 x g for 10 min at 4 C.
5. Discard the supernatant.
6. Wash the nuclei pellet once with cold 10 mM Tris-HCl (pH7.5), 10 mM NaCl.

Extraction of Nuclei

1. Suspend the isolated nuclei in 50-100 µL of extraction buffer.
2. Sonicate for 30 seconds.
3. Stand on ice for 30 min.
4. c.f.g. at 20,000 x g for 10 min.
5. Take supernatant (the crude nuclear extract).
6. Determine protein conc. by Bradford method or equivalent.
7. Store the crude nuclear extract at -70°C until use.

Note: Do not use any kind of protease inhibitor!!

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SIRT1 activity in an immunoprecipitate

Immunoprecipitation Followed by Measuring SITR1 Activity Protocol

Solutions and Reagents

Note: Prepare solutions with Milli-Q or equivalently purified water.

Cell Lysis Buffer (1X): 20 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM DTT.

Protein A Agarose Beads: Add 5 mL of 1X PBS to 1.5 g of Protein A Agarose Beads. Shake 2 hours at 4°C; spin down. Wash the pellet twice with PBS. Resuspend beads in 1 volume of PBS. (Can be stored for 2 weeks at 4°C)

Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing test compound for desired time.
2. To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 mL 1X ice-cold Cell Lysis Buffer to each plate (10 cm dish) and incubate the plate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate 4 times for 5 seconds each on ice.
6. Microcentrifuge for 10 minutes at 4°C, and transfer the supernatant to a new tube. The supernatant is the cell lysate. If necessary, lysate can be stored at –70°C.

Immunoprecipitation

1. Take 200 µL cell lysate and add anti-SIRT1 antibody (CY-P1016; 1-2 µg; incubate with gentle rocking for 2 hrs or overnight at 4°C.
2. Add protein A agarose beads (20 µL of 50% bead slurry). Incubate with gentle rocking for 1–3 hours at 4°C.
3. Microcentrifuge for 30 seconds at 4°C. Wash pellet 3 times with 500 µL of 1X Cell Lysis Buffer and with SIRT1 assay buffer (50 mM Tris-HCl (pH 8.8), 0.5 mM DTT). Keep on ice during washes.
4. After immunoprecipitation, add reaction mixture containing Fluoro-Substrate peptide solution to protein A agarose beads and measure NAD dependent deacetylase activity according to the procedure in CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit [Cat# CY-1151].

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Example of Test Results

Fig.1 Dose dependency curve of recombinant SIRT1 activity

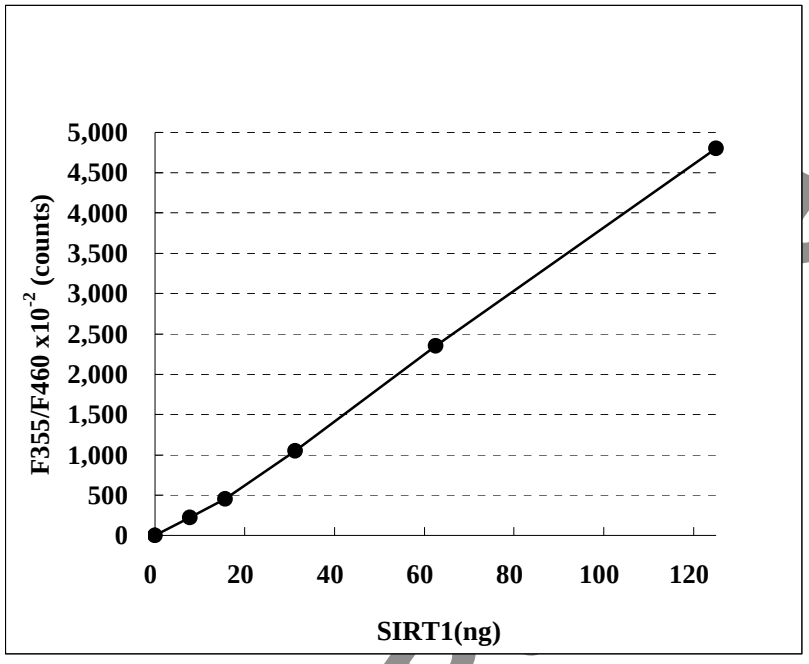

Fig.2 Time course of SIRT1-substrate deacetylation by recombinant SIRT1

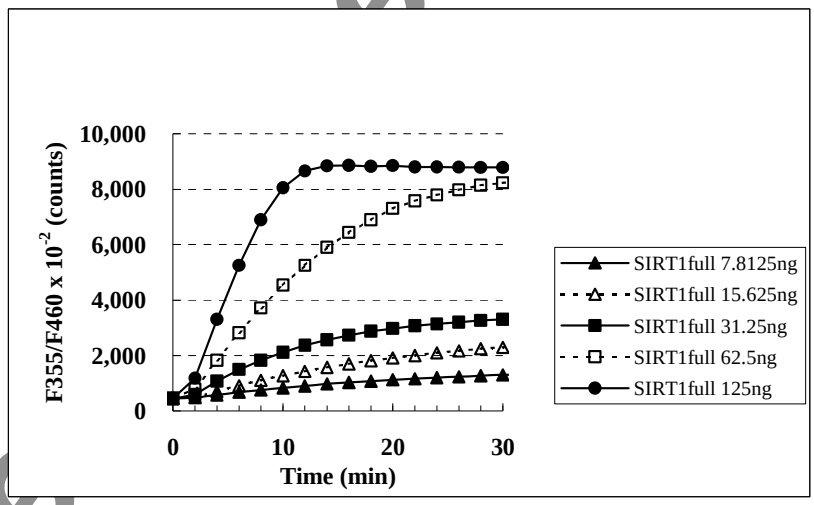

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Fig.3 Effect of Trichostatin A and NAD on recombinant SIRT1 activity

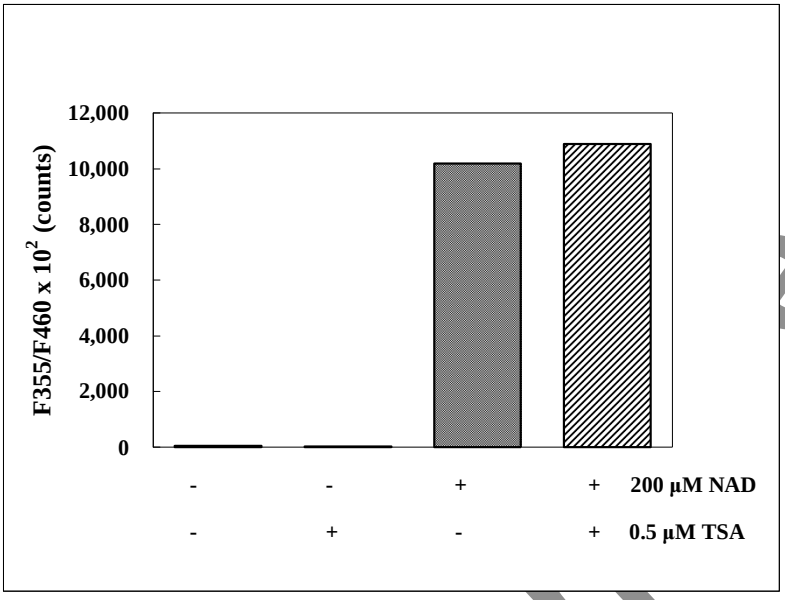

Fig.4 Km value of recombinant SIRT1 for Fluoro-Substrate Peptide

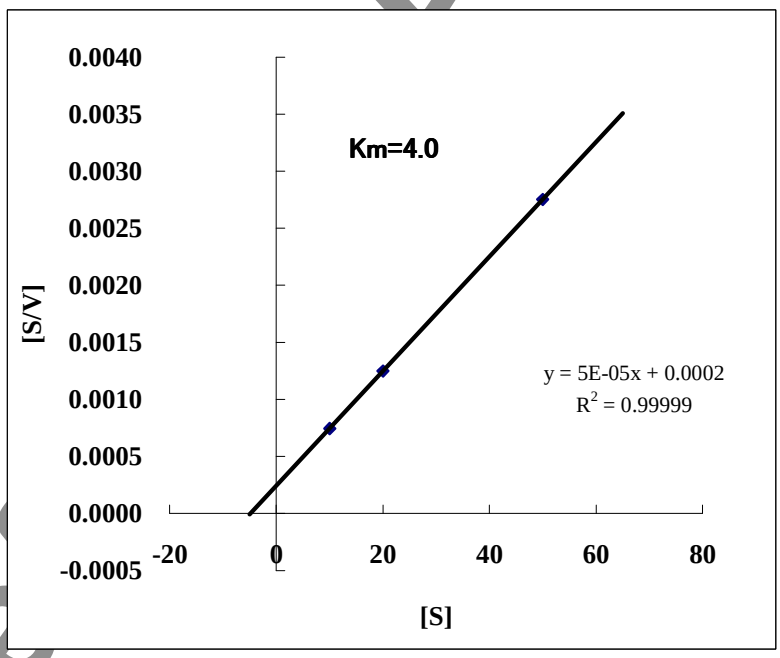

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Fig.5 Substrate Preference of HDAC and SIRT1

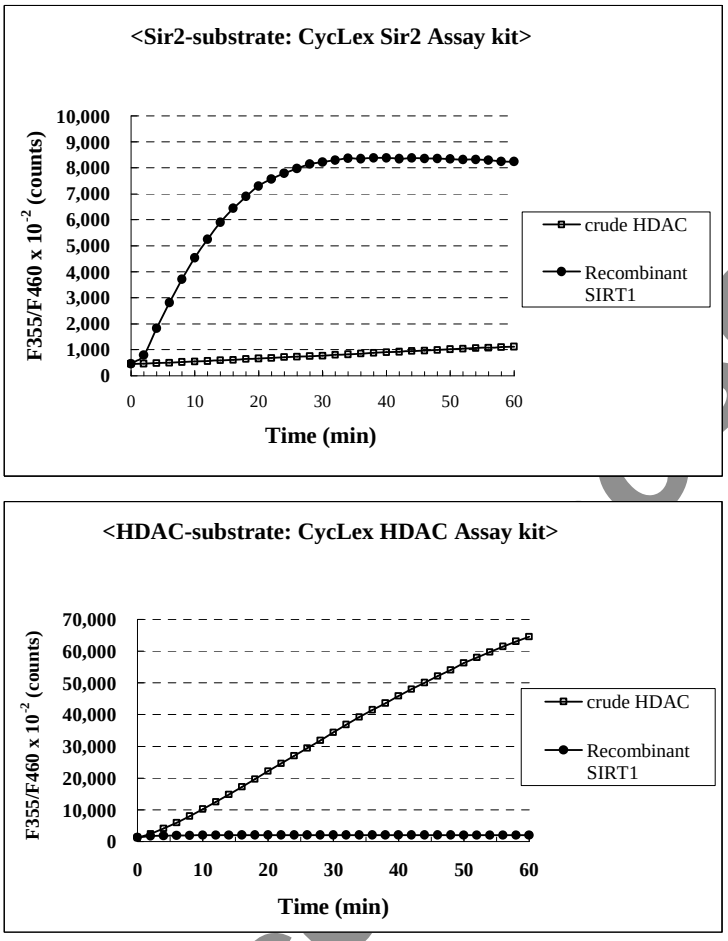

Fig.6 Stability of Fluorescence Intensity after Stop the Reaction

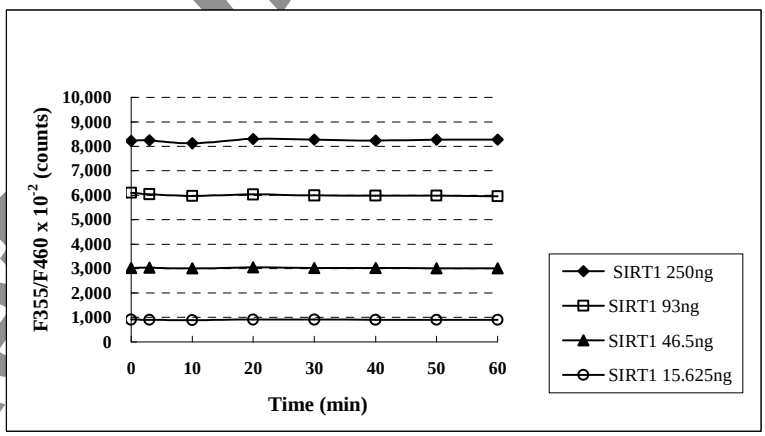

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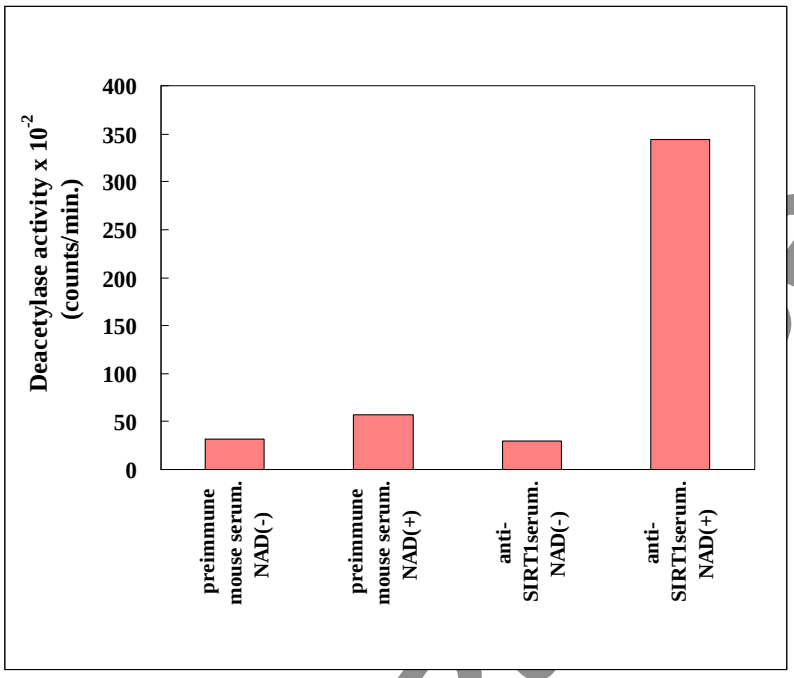
Fig.7 Measurement of 293T cell endogenous SIRT1 activity in an immunoprecipitate using anti-SIRT1 antibody (CY-P1016).



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Related Products

*** CycLex Cellular Histone Acetylation Assay Kit: Cat# CY-1140**
*** CycLex HDACs Deacetylase Fluorometric Assay Kit: Cat# CY-1150**
*** CycLex HDAC8 Deacetylase Fluorometric Assay Kit: Cat# CY-1158**
*** CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit: Cat# CY-1151**
*** CycLex SIRT2 Deacetylase Fluorometric Assay Kit: Cat# CY-1152**
*** CycLex SIRT3 Deacetylase Fluorometric Assay Kit: Cat# CY-1153**
*** CycLex SIRT6 Deacetylase Fluorometric Assay Kit: Cat# CY-1156**
*** Anti-Acetylated Histone/p53-K382 Mouse Monoclonal Antibody: Cat# CY-M1029**
*** Anti-Histone Deacetylase 1 (HDAC1) Rabbit Polyclonal Antibody: Cat# CY-P1011**
*** Anti-Histone Deacetylase 2 (HDAC2) Rabbit Polyclonal Antibody: Cat# CY-P1012**
*** Anti-Human SIRT1 Rabbit Polyclonal Antibody: Cat# CY-P1016**
*** NAD(+)-Dependent Deacetylase SIRT1: Cat# CY-E1151**
*** NAD(+)-Dependent Deacetylase SIRT2: Cat# CY-E1152**
*** NAD(+)-Dependent Deacetylase SIRT3: Cat# CY-E1153**
*** NAMPT (Nicotinamide Phosphoribosyltransferase): Cat# CY-E1251**
*** NMNAT1 (Nicotinamide Mononucleotide Adenylyltransferase 1): Cat# CY-E1252**

Note:
This product is covered under CycLex's patents.
U.S. Patent No. 7,033,778 and No. 7256013
European Patent No. 1243658
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